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Sensitivity to abscisic acid of guard-cell K^+ channels is suppressed by *abi1-1*, a mutant *Arabidopsis* gene encoding a putative protein phosphatase

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ABSTRACT **Abscisic acid (ABA) modulates the activities of three major classes of ion channels—inward- and outward-rectifying K^+ channels ($I_{K,in}$ and $I_{K,out}$, respectively) and anion channels—at the guard-cell plasma membrane to achieve a net efflux of osmotica and stomatal closure. Disruption of ABA sensitivity in wilty *abi1-1* mutants of *Arabidopsis* and evidence that this gene encodes a protein phosphatase suggest that protein (de-)phosphorylation contributes to guard-cell transport control by ABA. To pinpoint the role of ABI1, the *abi1-1* dominant mutant allele was stably transformed into *Nicotiana benthamiana* and its influence on $I_{K,in}$, $I_{K,out}$, and the anion channels was monitored in guard cells under voltage clamp. Compared with guard cells from wild-type and vector-transformed control plants, expression of the *abi1-1* gene was associated with 2- to 6-fold reductions in $I_{K,out}$ and an insensitivity of both $I_{K,in}$ and $I_{K,out}$ to 20 μM ABA. In contrast, no differences between control and *abi1-1* transgenic plants were observed in the anion current or its response to ABA. Parallel measurements of intracellular pH (pH_i) using the fluorescent dye 2′,7′-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF) in every case showed a 0.15- to 0.2-pH-unit alkalinization in ABA, demonstrating that the transgene was without effect on the pH_i signal that mediates in ABA-evoked K^+ channel control. In guard cells from the *abi1-1* transformants, normal sensitivity of both K^+ channels to and stomatal closure in ABA was recovered in the presence of 100 μM H7 and 0.5 μM staurosporine, both broad-range protein kinase antagonists. These results demonstrate an aberrant K^+ channel behavior—including channel insensitivity to ABA-dependent alkalinization of pH_i—as a major consequence of *abi1-1* action and implicate ABI1 as part of a phosphatase/kinase pathway that modulates the sensitivity of guard-cell K^+ channels to ABA-evoked signal cascades.**

Abscisic acid (ABA) participates in the growth and development of higher plants by controlling seed maturation and dormancy and signals conditions of water stress in vegetative tissues (1, 2). The hormone accumulates in the leaves during drought stress, evoking stomatal closure to reduce transpirational water loss (1). Stomatal closure is achieved through osmotic solute efflux from the guard cells, notably of K^+ from the vacuole and cytoplasm across the plasma membrane, and the consequent decline in guard-cell turgor. ABA initiates this flux by modulating three classes of ion channels—inward- and outward-rectifying K^+ channels ($I_{K,in}$ and $I_{K,out}$, respectively) and anion channels—events coordinated by at least two independent signal cascades that lead to a rise in cytoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and intracellular pH (pH_i) (1, 3, 4).

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Protein phosphorylation has also been implicated from studies of the ABA-insensitive 1 (*abi1*) mutant that interferes with ABA responsiveness in *Arabidopsis thaliana*. Mutations at this locus evoke a range of ABA-related phenotypes, notably aberrant control of stomatal aperture and consequent tendency to wilt (2, 5). The predicted ABI1 protein is related to type 2C protein serine/threonine phosphatases (PP2Cs) and, additionally, includes a putative Ca^{2+}-binding (EF-hand) site (6, 7). Hence, the observations implicate protein phosphatase, and by inference protein kinase activities, in ABA-mediated control of guard-cell membrane transport.

To establish the relationship of *ABI1*, ABA-evoked signaling, and control of the ion channels facilitating stomatal closure, we transformed the *abi1-1* dominant mutant gene into the diploid tobacco *Nicotiana benthamiana*. We report here that the transgene induced a complementary subset of mutant phenotypes, including the tendency to wilt, suggesting that elements in the *ABI1* signal cascade(s) are conserved across species, and we provide evidence linking ABI1 function to modulation of guard-cell K^+ channel sensitivity to ABA.

MATERIALS AND METHODS

Plant Material, Transformation, and Analysis. An *Arabidopsis* 6.5-kb genomic fragment containing the *abi1-1* mutant gene and cis-acting regulatory sequences was cloned into the *Bam*HI site of the binary pDE1000 T-DNA vector that carries a kanamycin-resistance marker (Plant Genetic Systems, Gent, Belgium). The resulting pDEBam6.5 construct (6) and the empty pDE1000 vector were introduced into *Agrobacterium tumefaciens* C58C1Rif(pGV2260) and used to transform leaf disks of *N. benthamiana*. Primary regenerants (T_0) and transgenic T_1 plants were selected by germination on 0.5 × GM agar plates supplemented with kanamycin sulfate (50 μg/ml) (8). Plants were grown at 22°C under a 16-h photoperiod on 0.5 × GM agar in magenta containers or on soil.

Stomatal apertures were recorded from stomata in epidermal strips under continuous superfusion (below) at ×320 magnification by using a charge-coupled device camera with transmitted light at >750 nm (Schott, Mainz, Germany). Northern and Southern blot analyses were carried out as described (9, 10) by using a *Sma* I subfragment (nt 239–1926) of *Arabidopsis ABI1* cDNA (6) as a probe. Filters were hybridized at 65°C and final washes were at 55°C in 0.1% SDS/15 mM NaCl/1.5 mM sodium citrate, pH 7.0.

Abbreviations: ABA, abscisic acid; $I_{K,in}$, inward-rectifying K^+ (channel) current; $I_{K,out}$, outward-rectifying K^+ (channel) current; pH_i, intracellular pH; BCECF, 2′,7′-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein; $[Ca^{2+}]_i$, cytoplasmic free $[Ca^{2+}]$; PP2C, type 2C protein serine/threonine phosphatase; *I–V*, current–voltage; $V_{1/2}$, half-maximal activation voltage.

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Electrophysiology and Photometry. Epidermal peels containing the guard cells were mounted and bathed in 5 mM Ca-Mes (pH 6.1; $[Ca^{2+}] \approx 1$ mM) with 10 mM KCl (3). ABA (racemic, Sigma) was included as required. Kinase antagonists H7 and staurosporine (Calbiochem) were prepared in dimethyl sulfoxide or ethanol and diluted at least 1:1000 in bath solution for use. Electrical recordings were carried out by using two- and four-barreled microelectrodes that, unless noted, were filled with 200 mM KOAc (3). Membrane currents were measured by voltage clamp under microprocessor control (μLAB, WyeScience, Wye, Kent, U.K.) by using three-pulse protocols (sampling frequency, 2 kHz) and bipolar staircase duty cycles (3).

$[Ca^{2+}]_i$ and pH_i were determined by ratio fluorescence with a Cairn microphotometer (Cairn, Faversham, U.K.) by using the dyes fura-2 and 2′,7′-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF; Molecular Probes) excited at 350 and 380 nm and at 440 and 490 nm (Schott, 10-nm half bandwidths), respectively, and were calibrated *in vivo* and *in vitro* (11). Fluorescence was recorded through a slit diaphragm after filtering with 520-nm longpass or 535-nm interference filters (Schott) and excluded microelectrode fluorescence. Dye loading was by iontophoresis (3) and was judged successful by visual checks for cytoplasmic dye distribution and by stabilization of the fluorescence ratio signal.

RESULTS

Phenotypic Characteristics of *abi1-1* Transformants. Compared to control plants, both wild-type and transformed with the T-DNA vector alone, *abi1-1* transgenic *N. benthamiana* displayed characteristics reminiscent of the original *Arabidopsis* abi1 phenotype (5). Of the 12 independent primary transformants obtained, 2 did not survive greenhouse conditions (22°C, 60% relative humidity) and 1 was sterile as a result of premature desiccation. Kanamycin-resistant plants were selected from the T_1 progeny derived from 8 of the 9 remaining transformants (Nba3–Nba8, Nba10, and Nba11). Each transgenic line exhibited a wilty phenotype (Fig. 1*A*) that correlated with expression of the *abi1-1* transcript (Fig. 1*B*), with the exception of Nba11, which also failed to show expression of the *abi1-1* transcript. [The *Arabidopsis ABI1* probe did not detect any putative *N. benthamiana* mRNA homologs (Fig. 1*B*).] The *abi1-1* transgene also reduced seed dormancy (compare refs. 5 and 6). Vivipary was observed in lines Nba3, Nba6–Nba8, and Nba10 with 8–86% of T_1 seed (segregating for the transgene) germinating in pod, and large proportions of remaining seed (Nba3, 74%; Nba7, 87%) germinated on imbibing at 4°C in the dark, conditions under which seed from wild-type plants failed to germinate (data not shown).

***abi1-1* Selectively Reduces Current Through Outward-Rectifying K^+ Channels.** Membrane currents were recorded under voltage clamp from guard cells of lines Nba5, Nba7, and Nba8 and control plants. Each plant was checked to verify the presence (*abi1-1* transformants) or absence (wild-type and vector transformants) of the *abi1-1* transgene by Southern blot hybridization (data not shown). The electrical characteristics of guard cells from the control plants ($n = 18$) were dominated by two distinct K^+ channel currents (1). Clamping the membrane either to voltages positive of the K^+ equilibrium potential ($E_K \approx -80$ mV in 10 mM K^+_o) or to voltages negative of ≈ -120 mV revealed time- and voltage-dependent currents that deactivated on clamping to -30 mV (Fig. 2 *A* and *B*). Tail current analyses for both $I_{K,in}$ and $I_{K,out}$ yielded reversal potentials that followed E_K as $[KCl]_o$ was varied, consistent with high K^+ selectivities (estimated $P_{Cl}/P_K < 0.02$), and both currents were blocked by 10 mM tetraethylammonium chloride (data not shown), a classic K^+ channel antagonist (1). Unlike *Vicia*, in the tobacco guard cells, $I_{K,out}$ showed a partial time- and voltage-dependent inactivation (Fig. 2*A*).

By comparison, voltage-clamp recordings from guard cells of *abi1-1* transgenic plants ($n = 27$) showed 2 (Nba7)- to 6 (Nba5)-fold reductions in $I_{K,out}$, but no significant differences were observed in $I_{K,in}$ (Fig. 2 *C* and *D*, also see Fig. 4). The effect of the transgene on $I_{K,out}$ was voltage-independent; no appreciable difference was evident in the kinetics for $I_{K,out}$ activation nor in its half-maximal activation voltage ($V_{1/2}$; compare Fig. 2 *B Upper Inset* and *D Upper Inset*). However, a purely scalar reduction in $I_{K,out}$ could be ruled out, since the time-dependent inactivation of $I_{K,out}$ was greatly reduced in the transgenic lines (compare Fig. 2 *A* and *C*).

***abi1-1* Interferes with K^+ Channel Response to ABA.** The predominant feature of all three transgenic lines was a severe attenuation or loss of K^+ channel response to ABA. No appreciable effect of ABA on current activation kinetics was found (Fig. 2*C*), and the current–voltage (*I–V*) relations showed only 10% inactivation of $I_{K,in}$ and virtually no activation of $I_{K,out}$ (Fig. 2*D*). Comparable results were obtained from all 18 Nba7, 5 Nba5, and 4 Nba8 cells challenged with ABA (Figs. 3 and 4). Indeed, on a cell-by-cell basis adding ABA generally led to a decline in current through $I_{K,out}$ in all three transgenic lines (for Nba7, 45 ± 11%, masked by statistical variation in Fig. 4).

In contrast, in control guard cells, ABA evoked a pronounced inactivation of $I_{K,in}$ and activation of $I_{K,out}$ (Fig. 2 *A*

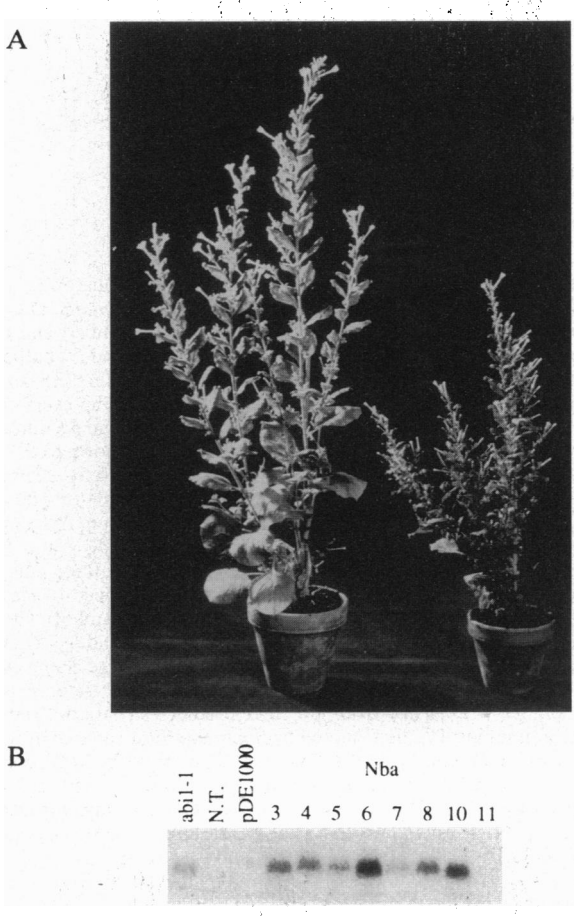

FIG. 1. Phenotypic and molecular characterization of *N. benthamiana* plants transformed with *abi1-1*. (*A*) Morphology of greenhouse-grown Nba11 (*Left*) and Nba7 (*Right*) transgenic plants. Except for kanamycin resistance, Nba11 plants were indistinguishable from wild-type or vector-transformed *N. benthamiana* (data not shown), whereas Nba7 and the other transgenic lines (data not shown) were smaller and wilty. (*B*) Northern blot analysis of *abi1-1* gene expression. Total RNA (10 μg) isolated from leaves of the original *Arabidopsis abi1* mutant (*abi1-1*), nontransformed wild type (N.T.), vector-transformed (pDE1000) *N. benthamiana*, and the indicated *abi1-1* transgenic lines (Nba3–Nba11) was hybridized to an *ABI1* cDNA probe.

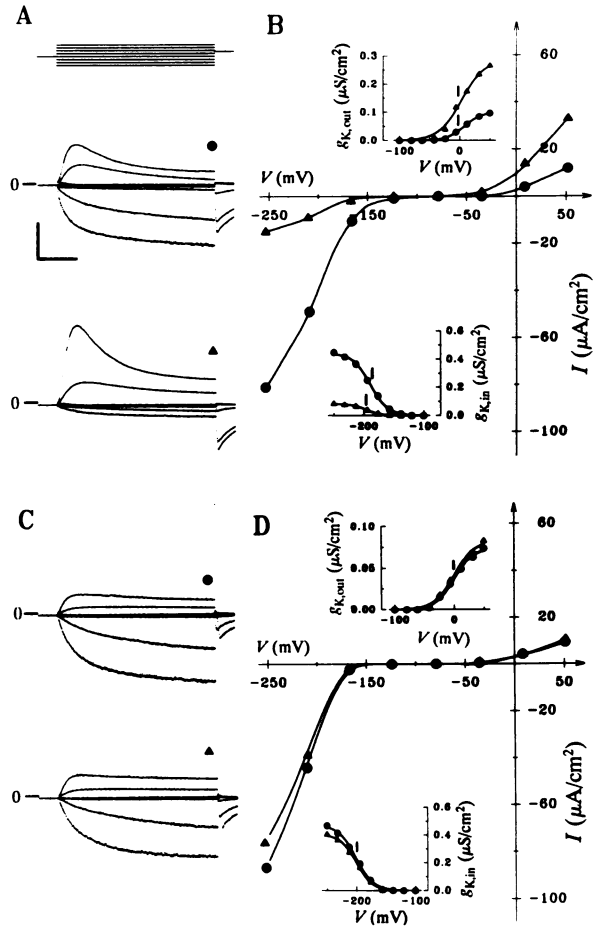

FIG. 2. Influence of the *Arabidopsis abi1-1* transgene on $I_{K,in}$ and $I_{K,out}$ currents and their response to ABA in *N. benthamiana*. Data are from a guard cell of a vector-transformed plant (*A* and *B*) and from a guard cell of the *abi1-1*-transformed line Nba7 (*C* and *D*) bathed in 5 mM Ca-Mes (pH 6.1) with 10 mM KCl. Cell parameters (3): surface area, 2.9×10^{-5} cm^2; volume, 5.6 pl. (*A*) Voltage clamp cycle (*Top*) and current trajectories recorded before (*Middle*) (●) and 6.5 min after (*Bottom*) (▲) adding 20 μM ABA. Current zeros are shown to the left. Clamp voltages: conditioning, −100 mV; test (8), +50 to −250 mV; tailing, −30 mV. Scale bar in *A*: vertical, 50 μA/cm^2 or 500 mV; horizontal, 1 s. Note the time-dependent inactivation of $I_{K,out}$ at positive voltages. (*B*) Steady-state *I–V* relations for $I_{K,in}$ and $I_{K,out}$ taken from currents recorded at the end of the test voltage pulses in *A* after subtracting the instantaneous currents recorded <4 ms into pulses at each test voltage (cross-referenced to *A* by symbol). (*Insets*) Conductance (g_K)–voltage relations for $I_{K,out}$ (*Upper*) and $I_{K,in}$ (*Lower*) determined as $g_K = I_K/(V - E_K)$ from additional voltage clamp cycles carried out before and 7 min into ABA exposure (cross-referenced by symbol). $E_K = -71$ mV from tail current analyses (data not shown). Vertical lines are $V_{1/2}$ determined by nonlinear least squares fitting to a Boltzmann function (11). $V_{1/2}$ for $I_{K,out}$ (*Upper*): −3 ± 2 mV, control (●); −5 ± 3 mV, +ABA (▲). $V_{1/2}$ for $I_{K,in}$ (*Lower*): −181 ± 3 mV, control (●); −196 ± 3 mV, +ABA (▲). (*C*) Current trajectories as in *A* recorded before (*Upper*) (●) and 11 min after (*Lower*) (▲) adding 20 μM ABA. Voltage clamp cycle and scales are as in *A*. (*D*) Steady-state *I–V* relations for $I_{K,in}$ and $I_{K,out}$ taken from current recorded at the end of the test voltage pulses in *C* as in *B* (cross-referenced to *C* by symbol). (*Insets*) Conductance (g_K)–voltage relations for $I_{K,out}$ (*Upper*) and $I_{K,in}$ (*Lower*) as in *B*. $E_K = -81$ mV from tail current analyses (data not shown). (*Upper*) $V_{1/2}$ for $I_{K,out}$: −3 ± 3 mV, control (●); −3 ± 2 mV, +ABA (▲). (*Lower*) $V_{1/2}$ for $I_{K,in}$: −198 ± 4 mV, control (●); −197 ± 3 mV, +ABA (▲).

and *B*) as in *Vicia* (3). Inactivation of $I_{K,in}$ was complete within 2–3 min, showed an appreciable voltage dependence as marked by the negative shift in $V_{1/2}$ (Fig. 2*B Lower Inset*), and yielded a mean 82 ± 6% inactivation at −250 mV (Fig. 4).

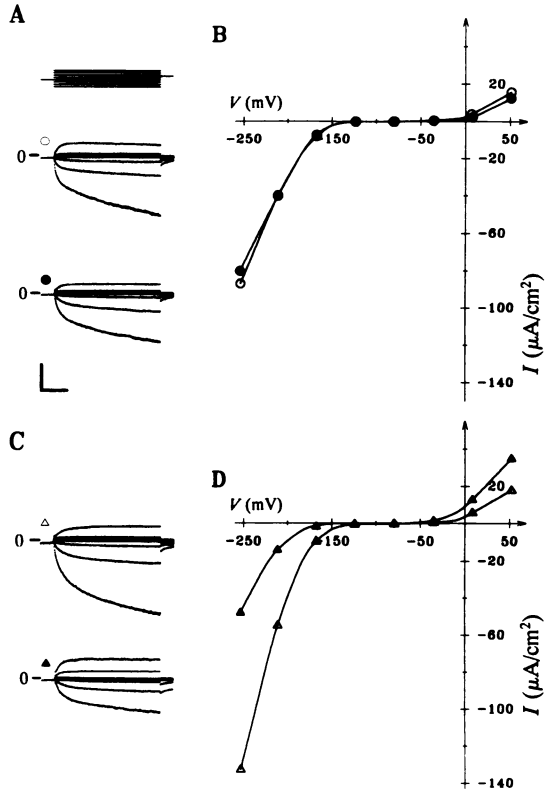

FIG. 3. Restoration of K^+ channel sensitivity to ABA by protein kinase antagonists. Data from one *N. benthamiana* guard cell, line Nba7, carrying the *Arabidopsis abi1-1* transgene, bathed in 5 mM Ca-Mes (pH 6.1) with 10 mM KCl before (*A* and *B*) and after (*C* and *D*) a 16-min exposure to 100 μM H7. (*A*) Voltage clamp cycle (*Top*) and current trajectories recorded before (*Middle*) (○) and 9.2 min after (*Bottom*) (●) adding 20 μM ABA. Current zeros are on the left. Clamp voltages and scale are as in Fig. 2. (*B*) Steady-state *I–V* relations for $I_{K,in}$ and $I_{K,out}$. Data were calculated as in Fig. 2 and curves are cross-referenced to *A* by symbol. (*C*) Current trajectories as in *A* recorded before (*Upper*) (△) and 4.5 min after (*Lower*) (▲) adding 20 μM ABA. Voltage clamp cycle and scales are as in *A*. (*D*) Steady-state *I–V* relations for $I_{K,in}$ and $I_{K,out}$ taken from current recorded at the end of the test voltage pulses in *C* as in *B*. Curves are cross-referenced to *C* by symbol. Quantitatively comparable results were obtained in experiments with four guard cells of the Nba7 and Nba5 transgenic plants using 0.5 μM staurosporine in place of H7. Neither kinase antagonist affected the K^+ currents in control guard cells.

Activation of $I_{K,out}$ in all but four cells was complete within 5–8 min of exposure to ABA and was without effect on $V_{1/2}$, giving a scalar increase in conductance (Fig. 2*B Upper Inset*). Mean currents for $I_{K,out}$ at +50 mV, on a cell-by-cell basis, showed a 2.1-fold ± 0.3-fold activation. [Anion current (6 ± 1 μA/cm^2 at −60 mV, $n = 18$) was also evident and was isolated in the presence of K^+ channel blockers CsCl and tetraethylammonium chloride (1, 12, 13). No significant difference in ABA-dependent activation of this current was found between control (2.3-fold ± 0.4-fold activation, $n = 18$) and *abi1-1* transformed plants (pooled, 2.5-fold ± 0.3-fold activation, $n = 27$).]

$[Ca^{2+}]_i$ and pH_i Are Unaffected by *abi1-1*. ABA control of guard-cell K^+ channels is normally mediated through an increase in pH_i and an independent increase in $[Ca^{2+}]_i$ (1, 3, 4, 14, 15). Activation of $I_{K,out}$ is insensitive to $[Ca^{2+}]_i$ (4) but is regulated by cytoplasmic alkalinization in ABA (3) as is $I_{K,in}$ (3, 11). Comparable pH_i sensitivities were found for both K^+ currents in wild-type *N. benthamiana* (M.R.B. and A.G., unpublished data). Thus, the loss of ABA response in the *abi1-1* transformants raised a question about transgene influence on the pH_i signal.

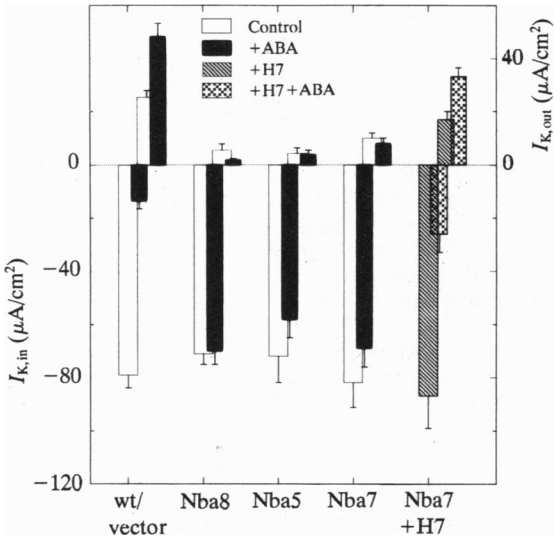

FIG. 4. Summary of the *Arabidopsis abi1-1* transgene influence on $I_{K,in}$ and $I_{K,out}$ K^+ channel currents and their response to ABA in *N. benthamiana* lines Nba5 (n = 5), Nba7 (n = 18), and Nba8 (n = 4). Data for wild-type and vector-transformed plants were equivalent and have been pooled (n = 18). Results are included for Nba7 in 100 μM H7 (n = 5). Current statistics are the mean ± SEM from cells bathed in 5 mM Ca-Mes (pH 6.1) with 10 mM KCl/±20 μM ABA. Standard clamp voltages: −250 mV, $I_{K,in}$; +50 mV, $I_{K,out}$. Differences (−ABA) between control and all transgenic lines for $I_{K,out}$ are significant at the 95% confidence level. The difference ±H7 (−ABA) for $I_{K,out}$ of Nba7 is significant only at the 70% confidence level.

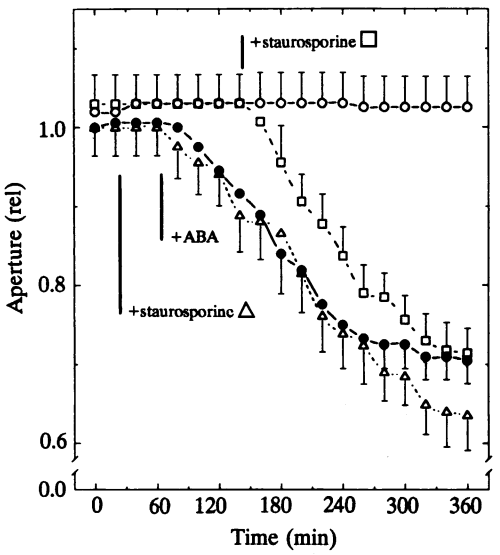

FIG. 5. Insensitivity of stomatal response to 20 μM ABA in epidermal peels from *N. benthamiana* carrying the *Arabidopsis abi1-1* transgene (○, □, and △). Data from control (wild-type, ●) *N. benthamiana* are included. Peels were bathed in 5 mM Ca-Mes (pH 6.1) with 10 mM KCl. ABA (all treatments) and 0.5 μM staurosporine (□ and △) were added at times indicated. Data are the mean ± SEM of tagged sets of 12–15 stomata (3) and have been normalized (○, □ with offset for clarity) to starting apertures for comparison. Mean starting apertures: wild-type, 6.6 ± 0.3 μm (●); Nba7, 10.4 ± 0.4 μm (○), 14.2 ± 0.5 μm (△), and 7.9 ± 0.3 μm (□). Experiments were repeated in triplicate, also with 100 μM H7, and gave equivalent results. Neither H7 nor staurosporine affected stomatal apertures or their response to ABA in wild-type *N. benthamiana* (data not shown).

To address this issue, guard cells were injected with the pH-sensitive dye BCECF and pH_i was recorded by fluorescence ratio microphotometry. Remarkably, no difference was observed between guard cells from the *abi1-1* transgenic and control plants (Table 1), ruling out any direct interference of *abi1-1* gene product with the pH_i signal. Resting pH_i in each case was close to 7.3, comparable to BCECF fluorescence ratio (11) and H^+-selective microelectrode measurements (3) from *Vicia* guard cells; and pH_i rose in ABA by ≈0.2 unit over 3–5 min in guard cells of both control and *abi1-1*-transformed plants (compare with ref. 3). Measurements using the Ca^{2+}-sensitive dye fura-2 failed to resolve any ABA-evoked changes in $[Ca^{2+}]_i$ either from the *abi1-1* transformed or control plants.

Protein Kinase Antagonists Restore ABA-Mediated Control of K^+ Channel Activities. Because the *abi1-1* mutation maps to the PP2C-like domain (6, 7), the mutant abi1-1 protein might impare protein dephosphorylation essential to K^+-channel control by ABA. Thus, a second question arose whether normal channel activity and response to ABA might be recovered by redressing an imbalance in protein phosphorylation by using protein kinase antagonists.

Table 1. pH_i and its response to 20 μM ABA in *N. benthamiana* are unaffected by the *abi1-1* gene in the transgenic lines Nba7 and Nba8

	pH_i		
Cell lineage (n)	Control	+ ABA	δpH_i
wt/vector (7)	7.3 ± 0.1	7.5 ± 0.1	0.21 ± 0.02
Nba7 (11)	7.35 ± 0.06	7.59 ± 0.07	0.23 ± 0.03
Nba8 (4)	7.3 ± 0.2	7.6 ± 0.1	0.17 ± 0.05

pH_i measured by BCECF fluorescence ratio microphotometry after loading by microinjection. Excitation wavelengths were 440 and 490 nm; emission was recorded at 535 nm. δpH_i is the change in pH_i ± ABA on a cell-by-cell basis. Data from wild-type *N. benthamiana* and plants transformed with the vector alone (wt/vector) were virtually identical and have been pooled.

To test this hypothesis, guard cells from the transgenic lines were challenged with ABA before and after pretreatment with H7 and staurosporine, both broad-range kinase antagonists (16). Data from one guard cell in Fig. 3 show that the current trajectories (Fig. 3 *A* and *C*) and *I–V* relations (Fig. 3 *B* and *D*) uncovered an ≈20% increase in $I_{K,in}$ and $I_{K,out}$ with exposure to 100 μM H7 alone, and the ABA sensitivities of both K^+ currents were recovered in the presence of the kinase antagonist. Comparable results were obtained in all five cells treated with H7, in four cells with 0.5 μM staurosporine, and when H7 was added in the presence of ABA (for Nba7, see Fig. 4). Recovery of the K^+ currents was paralleled by stomatal response to ABA. Fig. 5 shows that ABA promoted stomatal closure in wild-type *N. benthamiana* but not in Nba7 transformants, except in the presence of protein kinase antagonist.

DISCUSSION

The tendency to wilt and the reduced seed dormancy of *N. benthamiana* transformed with the *Arabidopsis abi1-1* transgene are reminiscent of the original *Arabidopsis abi1* phenotype (5) and of ABA-deficient mutants in *Arabidopsis* (17) and *Nicotiana plumbaginifolia* (18). That a putative *ABI1* homolog was not detected in *N. benthamiana* by Northern blot analysis may reflect a low transcript level but is also consistent with the wide structural divergence among PP2Cs (6, 7, 19). Thus, the abi1-1 protein appears to interfere with ABA signaling in *Arabidopsis* and tobacco via conserved mechanisms.

Electrophysiological analyses of *N. benthamiana* transformed with *abi1-1* argue strongly for a function of the gene product in modulating guard-cell K^+ channel sensitivity to ABA by protein phosphorylation. Each of the three transgenic lines examined showed reductions in background $I_{K,out}$ reminiscent of K^+ current behavior in the presence of protein phosphatase antagonists (20); alterations in $I_{K,out}$ inactivation

kinetics pointed to *abi1-1* interaction with K^+-channel gating; in each line the K^+ channels failed to respond normally to ABA, yet in no case was there an effect on the pH_i signal associated with $I_{K,out}$ control; finally, the sensitivity of both K^+ currents to ABA and stomatal closure were restored by kinase antagonists.

Indeed, the influence of the transgene on K^+ channel behavior is wholly consistent with stomatal disfunction and the wilty phenotype. The transport characteristics of *N. benthamiana* guard cells were found to parallel those of *Vicia* and *Commelina* for which $I_{K,out}$ is the predominant pathway for K^+ loss during stomatal closure (1). So, in itself, the reduction in background $I_{K,out}$ in the transgenic lines and its ABA insensitivity translate directly to a severe restriction in the capacity for K^+ efflux. The parallel insensitivity to ABA of $I_{K,in}$, a major pathway for K^+ uptake during stomatal opening (1), implies a corresponding failure to restrict the capacity for K^+ influx. Even so, net K^+ movement will be subject to transmembrane charge balance in the absence of the voltage clamp. Voltage clamp analyses ruled out an appreciable effect on anion channels, but any impact of the transgene on other currents, especially that of the H^+-ATPase, remains to be examined.

How then might the *abi1-1* transgene impare stomatal function? Remarkably, the *abi1-1* transgene was without effect on the pH_i alkalinization evoked by ABA (Table 1), despite its action in repressing the activation of $I_{K,out}$. Because changes in pH_i couple hormonal control to both K^+ currents (refs. 3, 11, and 21, and M.R.B. and A.G., unpublished data), the *abi1-1* transgene must interact in K^+ channel regulation downstream from the pH_i signal, plausibly through a separate transduction pathway. Furthermore, we note that the most pronounced effect of the *abi1-1* transgene was seen in $I_{K,out}$, which is nominally $[Ca^{2+}]_i$-insensitive in *Nicotiana* (M.R.B. and A.G., unpublished data) as it is in *Vicia* (1). So, while evidence for $[Ca^{2+}]_i$-mediated signaling was not forthcoming, arguably any $[Ca^{2+}]_i$ dependence to ABI1-mediated control of the K^+ channels is likely to appear secondarily, rather than precipitating stomatal closure directly on an ABA stimulus.

That ABI1 displays PP2C-like activity is supported both from biochemical assays and from partial functional complementation of a yeast PP2C mutant (ref. 7 and N. Bertauche, J.L., and J.G., unpublished data). The *abi1-1* mutation appears to confer a gain of function, since triploid *Arabidopsis* (*abi1-1/ABI1/ABI1*) display the mutant phenotypes (6) as do *abi1-1* transgenic *N. benthamiana*. One possibility, then, is that the mutant abi1-1 phosphatase interferes with the action of its wild-type counterpart, possibly sequestering and preventing substrate dephosphorylation. This explanation would account for restoration of ABA sensitivity by kinase antagonists (Figs. 3–5), implying that the antagonists act in parallel to redress a phosphorylation imbalance of ABI1 substrate(s). A simple interpretation would maintain that ABI1 is part of a kinase/phosphatase pair which interacts directly with the K^+ channels (22). An alternative, but not exclusive, interpretation would place the ABI1 target upstream within a signal cascade leading to K^+ channel control by ABA.

In conclusion, our findings demonstrate a profound action of the *abi1-1* gene, via a phosphorylation-sensitive pathway, on the K^+ channels of stomatal guard cells. The results highlight a dual action on steady-state activity and on the ABA sensitivity of the channels—notably of $I_{K,out}$, which facilitates K^+ efflux during ABA-induced stomatal closure—independent of ABA-evoked alkalinization of pH_i. Other classes of protein phosphatases (PP1/2A and PP2B) have been implicated in guard-cell K^+ channel control (20, 23). Their relationship to hormonal signaling *per se* remains speculative, however, and their action in modulating channel activity as well as their antagonist specificities (24) is clearly distinct from that of PP2Cs. An immediate issue now is how ABI1 and these several other kinases and phosphatases interrelate in modulating the guard-cell K^+ channels. Equally, a question hangs over ABI1 interaction with ABA-evoked signals, notably pH_i, and whether ABI1 targets the K^+ channels directly or via other regulatory proteins.

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